

(19)  **Canadian  
Intellectual Property  
Office**

An Agency of  
Industry Canada

**Office de la Propriété  
Intellectuelle  
du Canada**

Un organisme  
d'Industrie Canada

(11) **CA 2 297 345**

(13) **A1**

(43) 29.07.2000

(12)

(21) 2 297 345

(51) Int. Cl.<sup>7</sup>: **C12N 007/01, A61K 039/12,  
A61K 039/15**

(22) 27.01.2000

(30) 99200259.2 EP 29.01.1999

(71) **AKZO NOBEL N.V.,  
Velperweg 76  
NL-6824 BM, ARNHEM, XX (NL).**

(72) **VAN LOON, ADRIAAN ANTHONIUS WILHELMUS  
MARIA (NL).**

(74) **FETHERSTONHAUGH & CO.**

(54) **NOUVELLE CLASSE ANTIGENIQUE DE REOVIRUS AVIENS**

(54) **NOVEL ANTIGENIC CLASS OF AVIAN REOVIRUSES**

(57)

The present invention provides a new antigenic class of avian reoviruses which are involved in enteric disease conditions in poultry. Moreover, the invention provides a vaccine for use in the protection of poultry against such disease conditions derived from the new type of avian reoviruses.

Express Mail: EV 630723817 US  
Applicant: van de ZANDE et al.  
Application No: 10/749,907

**BEST AVAILABLE COPY**



(72) VAN LOON, ADRIAAN ANTHONIUS WILHELMUS MARIA, NL

(71) AKZO NOBEL N.V., NL

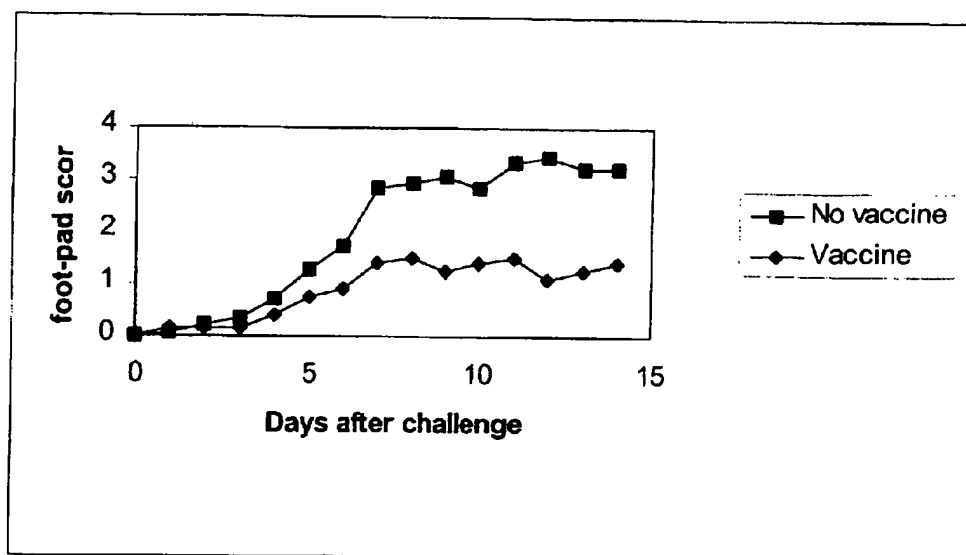
(51) Int.Cl.<sup>7</sup> C12N 7/01, A61K 39/15, A61K 39/12

(30) 1999/01/29 (99200259.2) EP

(54) **NOUVELLE CLASSE ANTIGENIQUE DE REOVIRUS AVIENS**

(54) **NOVEL ANTIGENIC CLASS OF AVIAN REOVIRUSES**

A: Protection evaluated by footpad lesions



(57) The present invention provides a new antigenic class of avian reoviruses which are involved in enteric disease conditions in poultry. Moreover, the invention provides a vaccine for use in the protection of poultry against such disease conditions derived from the new type of avian reoviruses.



ABSTRACT

The present invention provides a new antigenic class of avian reoviruses which are involved in enteric disease conditions in poultry. Moreover, the invention provides a vaccine for use in the protection of poultry against such disease conditions derived from the new type of  
5 avian reoviruses.

### Novel antigenic class of avian reoviruses

The present invention relates to an avian reovirus and a vaccine comprising an avian reovirus in a live attenuated or inactivated form.

5 Commercial broiler production evolved over the last several decades into an industry which is characterised by its high efficiency in the confinement rearing of the livestock. However, the strong tendency towards increasing of the efficiency of the rearing phase is not accomplished without encountering some inherent difficulties. Most notable is the increased incidence of infectious diseases that often occurs in high density, close confined animal  
10 populations. Many of the most devastating diseases in poultry have been limited or controlled by vaccination or by treatment with therapeutic agents such as antibiotics. Unfortunately, however, there are still a number of diseases of complex aetiology that have not been controlled with drugs and for which a suitable vaccination program is unavailable.

Since the late 1970s, the poultry industry has been confronted with such complex diseases  
15 in broiler chickens suffering from enteric problems. One of such diseases which results in a variety of disease conditions in affected chickens, including enteritis, is named for the main clinical sign and macroscopical observation: malabsorption syndrome (MAS). Alternatively, this disease is designated as infectious runting stunting syndrome, pale bird disease or brittle bone disease. Although a large number of assorted disease conditions are linked with MAS, in  
20 all cases poor growth and retarded feathering are observed. Additionally, a large variety of other signs and lesions, such as mortality, secretion of too liquid faeces and/or maldigested feed, pancreatic atrophy, proventriculitis, bone changes, thymic and bursal atrophy, etc. have been correlated with MAS.

Kouwenhoven et al. (Avian Pathology 17, 879-892, 1988) defined MAS by five criteria, i.e.

- 25 (i) growth impairment up to 3 weeks after infection of one-day-old chicks
- (ii) excretion of yellow orange mucoid to wet droppings
- (iii) increased plasma alkaline phosphatase (ALP) activity
- (iv) decreased plasma carotenoid concentration (PCC)
- (v) macroscopically widened epiphyseal growth plates of the proximal tibia.

30 Retarded growth in broilers becomes obvious by 1 week of age or earlier. From 5 to 20% of the birds in a flock may be affected and these birds will be half the size or less of their penmates by 4 weeks of age. Affected flocks have poor feed conversions and the intestines are pale and contain undigested feed.

Although the pathogenesis of MAS is poorly understood, the likely pathogenesis of this syndrome is the direct action of infectious agent(s) on the digestive tract and associated organs, which would also explain the recurring secretion of too liquid faeces and/or maldigested feed.

The syndrome described here results in a general lack of performance, including  
5 diminished weight gains, poor feed conversion and reduced marketability of the affected flocks. As a result of MAS the poultry industry suffers major economical losses annually. Therefore, the poultry industry is in need for a way to control MAS, such that one or more of the assorted disease conditions observed in broilers can be prevented.

Reoviruses are ubiquitous in poultry world-wide. Reoviruses have been found to be the  
10 causative agent of an arthritic condition affecting the major weight bearing joint capsules and tendon sheets in the legs, designated as viral arthritis/tenosynovitis.

In some reports reoviruses were also isolated from chickens displaying MAS associated disease conditions. In these reports it is speculated that the reoviruses have an etiological relationship with one or more of the MAS associated disease conditions, but firm proof of the  
15 involvement of reoviruses in MAS was not provided therein.

In van der Heide et al. (Avian Diseases 25, 847-856, 1981) a reovirus was isolated from the intestines of young broilers with clinical diarrhoea. Although this reovirus isolate was capable of inducing lesions of tenosynovitis and femoral head fractures and osteoporosis, this isolate did not consistently induce diarrhoea in chickens experimentally infected with the  
20 reovirus.

Page et al. (Avian Diseases 26, 618-624, 1982) isolated reoviruses from a flock experiencing lameness, stunting and erratic feather development. Although, the oral inoculation into susceptible broiler-type chickens produced a clear effect on weight gains, feather development and induced lesions in a number of organs, the induction of diarrhoea or wet letter  
25 was not reported.

Hieronymus et al. (Avian Diseases 27, 246-254, 1983) reported the isolation of several strains of reovirus from the intestines of chickens with suspected MAS and determined the antigenic relationship of these strains with reovirus strain S1133 which is commonly used as a vaccine strain for the control of infectious tenosynovitis. The authors confirm that despite the  
30 fact that the reoviruses were isolated from chickens with clinical MAS, it remained to be proven that the reoviruses were the causative agent of MAS.

Eidson et al. (Poultry Science 64, 2081-2086, 1985) investigated the effect of an inactivated reovirus vaccine, derived from the C08 strain isolated by Hieronymus et al., in broiler flocks experiencing problems with MAS as well as tenosynovitis. Although the vaccine had a positive effect on body weight of the broiler population, there was no difference in feed conversion observed. Furthermore, no effect of the vaccine on enteritis-associated disease conditions, such as wet litter was reported.

Rosenberger et al. (Avian Diseases 33, 535-544, 1989) also isolated several reovirus strains from the tendons and bone marrow of field-reared commercial broiler chickens. Although, chickens inoculated with the reovirus strains were examined for clinical disease, signs of diarrhoea or wet litter were not reported.

Kouwenhoven et al. (Avian Pathology 17, 879-892, 1988) also investigated the role of reovirus in the malabsorption syndrome. These authors could not reproduce MAS with reovirus isolated from a field case, and concluded that reovirus is not the primary etiological agent of MAS. It is speculated therein that infectious agents, including reoviruses and adenoviruses may act as some kind of trigger in the malabsorption syndrome.

However, in addition to the above-mentioned publications, many other viruses have been associated with MAS. These include rotaviruses, parvoviruses, enterovirus-like viruses, togavirus-like viruses, coronavirus-like viruses, adenoviruses and caliciviruses. Additionally, it was also suggested that bacteria be involved in the aetiology. MAS-like field syndromes have also been attributed to mycotoxins in the prior art and it is speculated that mycotoxins or other toxins should not be ignored as the causative agent of MAS.

In a recently published review (World Poultry 14, 57-58, 1998), McNulty summarised the state of the art on MAS. McNulty stressed the non-availability of a vaccine against MAS and stated that the virus isolations, as well as the virologic and microbiologic examinations of samples isolated from the field reported so far have not provided useful results with regard to the identification of the causative agent(s) of MAS. McNulty speculated that this approach is not likely to yield useful results. Instead, management measures in MAS affected production sites is considered to be the best weapon for control of MAS.

Hence, there still exists a need for a vaccine which induces an effective protection against certain enteric problems experienced by chickens, such as the enteric problems associated with MAS, resulting in the secretion of too liquid faeces and/or maldigested feed.

Moreover, avian reoviruses display considerable antigenic heterogeneity and the emerging of new antigenic classes of avian reoviruses may have important implications for the use of reovirus vaccines in poultry.

The present inventors have now identified a novel antigenic class of avian reoviruses.  
5 Furthermore, it is demonstrated that avian reoviruses belonging to this novel antigenic class are able to induce pronounced disease conditions which are also associated with MAS, such as secretion of too liquid faeces and/or maldigested feed and growth retardation.

Therefore, it is an object of the present invention to provide the causative agent of a MAS-like enteric disease condition belonging to a new antigenic class of avian reoviruses.

10 Another object of the present invention is to provide a vaccine which effectively affords protection in poultry against disease caused by avian reoviruses of the new antigenic class.

It is a further object of the present invention to provide a vaccine which effectively affords protection in poultry against an enteric disease condition which is also associated with MAS.

15 It has now been found that these objects can be met by providing an avian reovirus belonging to an antigenic class of avian reoviruses, characterised in that the avian reovirus belonging to the antigenic class is able to induce antiserum in an animal, which antiserum causes a reduction of the plaques formed by avian reovirus ERS, a sample of which is deposited at the ECACC, Salisbury, UK under accession no. 99011475, of at least 75% in a plaque  
20 reduction assay.

It has been observed that such avian reoviruses not only display hitherto unknown antigenic properties (Example 1, Table 2 and 3), but it has also been found that the avian reoviruses according to the present invention are able to induce the secretion of too liquid faeces and/or maldigested feed by a broiler chicken or in some cases may even lead to mortality. Hence, the  
25 new avian reovirus is designated herein as enteric reovirus strain (ERS).

The excretion of wet faeces in broiler flocks is one of the disease conditions which is also generally observed in MAS affected broilers in the field. Moreover, it is anticipated that this clinical disease condition is one of the causes of the most pronounced clinical signs in MAS affected broilers, i.e. that of growth retardation. In the examples it is shown that an avian  
30 reovirus according to the invention induces the excretion of wet faeces by broilers orally infected with the reovirus, i.e. around the cloaca of the birds clothing of pasting was observable.

The experiments in the examples also show that the oral infection of the new avian reovirus also results in a growth retardation in the infected broilers if compared with the control chickens.

5 The plaque reduction assay is an assay which is widely used in the art for determining the antigenic relationship between (avian reo)virus isolates (see e.g. Nersessian et al., Am. J. Vet. Res. 50, 1475-1480, 1989). Moreover, for the purpose of the present invention a detailed description of the plaque reduction assay is provided in Example 1. Obviously, the antiserum to be used in the plaque reduction assay should be of appropriate quality. Methods for the preparation of such antiserum are also described in Example 1.

10 Generally, appropriate antiserum raised against live avian reoviruses can be prepared by inoculating 3 to 4 weeks old SPF chickens subcutaneously or intramuscularly with a live virus strain having an infectious titre between  $10^{2.0}$  -  $10^{9.0}$  TCID<sub>50</sub>/animal; more preferably between  $10^{3.0}$  -  $10^{6.0}$  TCID<sub>50</sub>/animal. Blood can be collected 3 to 4 weeks after infection, preferably 4 weeks after infection. Chickens may also be re-infected with the same live virus strain 3 to 4 weeks after the first infection with approximately the same dose as used in the first infection. 15 Blood is collected between 2 and 4 weeks after the second infection.

Appropriate antiserum raised against inactivated avian reovirus strains can be obtained by inoculating 3 to 4 weeks old SPF chickens subcutaneously or intramuscularly with the inactivated virus preparation. The infectious titre of the preparation before inactivation may be 20 between  $10^{7.0}$  -  $10^{11.0}$  TCID<sub>50</sub>/animal; more preferably between  $10^{8.0}$  -  $10^{10.0}$  TCID<sub>50</sub>/animal. Blood can be collected 3 to 4 weeks after inoculation, preferably 4 weeks after inoculation. Chickens may also be re-inoculated with the same inactivated virus preparation 3 to 6 weeks after the first inoculation. Blood is collected between 2 and 4 weeks after the second inoculation.

25 The identification of the novel avian reovirus according to the invention allows the preparation of new avian reovirus vaccines which can effectively protect poultry against disease conditions resulting from the infection by the new antigenic class of avian reoviruses. In particular, the novel avian reoviruses allow the preparation of new avian reovirus vaccines which can effectively protect poultry against disease conditions such as the secretion of too 30 liquid faeces and/or maldigested feed and growth retardation. Such disease conditions are also associated with MAS.



The avian reovirus according to the invention can also be isolated from the field. An important aspect of the method of isolation is the identification of the target animal to be used as a starting-point for the virus isolation. A typical broiler to be used for this purpose shows the following signs: the secretion of too liquid faeces and/or maldigested feed resulting in growth retardation.

Subsequently, the intestine is isolated from the affected chicken followed by homogenisation of the organ in a suitable buffer. Thereafter, the homogenised tissue is centrifuged and the supernatant is filtrated through filters with a pore size of 0.2  $\mu\text{m}$ . A sample of the filtrate is added to freshly prepared primary chicken cells, preferably chicken embryo liver (CEL) cells, and 4-8 days after incubation the monolayers are inspected for the presence of a cytopathic effect (CPE). If no CPE is present a freeze/thawed suspension of the first monolayer is added to freshly prepared CEL cells. If after the first passage or the second passage CPE is observed, then the virus is further characterised by its in vivo properties in broilers to induce the secretion of too liquid faeces and/or maldigested feed and by its antigenic properties as determined in the plaque reduction assay or immuno-fluorescence-technique (IFT) using specific polyclonal- and monoclonal antibodies as described below.

A more detailed method for the isolation of an avian reovirus according to the present invention is disclosed in Example 1.

Although the intestines are used in the process for the isolation of the present avian reovirus as the preferred starting material, the avian reovirus can also be isolated from the liver of affected broiler chickens or from the faeces excreted by such a broiler chicken. It should also not be excluded that other organs may serve as the starting material for the isolation of the avian reovirus according to the invention.

In particular, the present invention provides an avian reovirus as described above, the induced antiserum against the avian reovirus being able to cause a reduction of the plaques formed by avian reovirus ERS, a sample of which is deposited at the ECACC, Salisbury, UK under accession no. 99011475, of at least 80 %, preferably at least 90%, in a plaque reduction assay.

In an even more preferred embodiment of the invention an avian reovirus is provided which displays a specific immunological reaction pattern with a specific panel of polyclonal antiserum and monoclonal antibodies (Moabs). This specific reaction pattern is different from  
5 that displayed by hitherto known avian reoviruses.

Monoclonal antibodies are useful for identifying characteristics of an infectious agent, and for determining similarities and differences among different isolates of the same or similar micro-organism. Vakharia et al. (Proceedings of the International Symposium on adenovirus  
10 and reovirus infections in poultry, Rauischholzhausen, Germany, 1996, 295-304) disclosed a panel of 9 anti-reovirus Moabs and tested avian reovirus field isolates for their reactivity with these Moabs. Different patterns of reactivity of the panel of Moabs allowed the classification of the isolates based on its relatedness to the pattern of reactivity.

In this embodiment of the invention an avian reovirus as described above is provided  
15 which is further characterised by (i) its reactivity in the IFT with a polyclonal antiserum raised against an avian reovirus isolate, preferably against the prototype reovirus strain 1133, and (ii) the absence of reactivity in the IFT with the Moabs INT 13-06, INT 14-11 and 15-01 INT (samples of which are deposited at the ECACC under accession no. 99011472, 99011473 and 99011474, respectively).

20 The avian reovirus according to the invention defined by the panel-pattern represents a novel antigenic type of reoviruses as demonstrated in Example 1 and Table 3. Despite the fact that many of the prior art reovirus strains shown in Table 3 are isolated from tissues of broiler chickens (including intestines) showing signs and lesions associated with MAS, avian reoviruses according to the present invention having the novel antigenic properties defined  
25 above have not been disclosed in the prior art.

An even more preferred avian reovirus according to the present invention is avian reovirus ERS (isolate 1) which is characteristic for avian reoviruses according to the present invention, a sample of which is deposited at the ECACC under accession no. 99011475.

The avian reovirus according to the invention can be in a live, live attenuated or  
30 inactivated form.

The invention provides in a further aspect a vaccine for use in the protection of poultry against disease conditions resulting from an avian reovirus infection, such as enteric disease conditions observed with MAS, comprising an avian reovirus according to the invention and a pharmaceutical acceptable carrier or diluent.

- 5       The avian reovirus according to the present invention can be incorporated into the vaccine as a live attenuated or inactivated virus. The property of the avian reovirus to induce MAS-associated disease conditions as described above are significantly reduced or completely absent if the avian reovirus is in a live attenuated or inactivated form.

      Attenuation of an avian reovirus according to the invention can be achieved by methods  
10 well known in the art for this purpose, such as disclosed in Gouvea et al. (*Virology* 126, 240-247, 1983). Briefly, after the isolation of the virus from a target animal, a virus suspension is inoculated onto primary chicken embryo fibroblasts (CEFs). If the isolate is not able to produce CPE, then the virus is passaged repeatedly (e.g. 3-10 times) until CPE is observed. As soon as CPE is visible, cells and cell culture fluids are collected, frozen and thawed, clarified by  
15 centrifugation and the supernatant containing the avian reovirus isolate is aliquoted and stored at - 20 °C. This process may be repeated (e.g. 10-100 times) to further attenuate the virus.

      A vaccine according to the invention can be prepared by conventional methods such as for example commonly used for the commercially available live- and inactivated reovirus vaccines. The preparation of veterinary vaccine compositions is inter alia described in  
20 "Handbuch der Schutzimpfungen in der Tiermedizin" (eds.: Mayr, A. et al., Verlag Paul Parey, Berlin und Hamburg, Germany, 1984) and "Vaccines for Veterinary Applications" (ed.: Peters, A.R. et al., Butterworth-Heinemann Ltd, 1993).

      Briefly, a susceptible substrate is inoculated with an avian reovirus according to the invention in a live or live attenuated form, and propagated until the virus replicated to a desired  
25 infectious titre or antigen mass content after which reovirus containing material is harvested and formulated to a pharmaceutical composition with prophylactic activity.

      Every substrate which is able to support the replication of the avian reoviruses defined above, if necessary after adaptation of the avian reoviruses to a substrate, can be used to produce a vaccine according to the present invention. Suitable substrates include primary  
30 (avian) cell cultures, such as chicken embryo liver cells (CEL), chicken embryo fibroblasts (CEF) or chicken kidney cells (CK), mammalian cell lines such as the VERO cell line or the BGM-70 cell line, or avian cell lines such as QT-35, QM-7 or LMH. Usually, after inoculation

of the cells, the virus is propagated for 3-10 days, after which the cell culture supernatant is harvested, and if desired filtered or centrifuged in order to remove cell debris.

Alternatively, the avian reovirus according to the invention can be propagated in embryonated chicken eggs followed by harvesting the avian reovirus material by routine  
5 methods.

The vaccine according to the invention containing the live attenuated virus can be prepared and marketed in the form of a (frozen) suspension or in a lyophilised form. The vaccine additionally contains a pharmaceutically acceptable carrier or diluent customary used for such compositions. Carriers include stabilisers, preservatives and buffers. Suitable  
10 stabilisers are, for example SPGA, carbohydrates (such as sorbitol, mannitol, starch, sucrose, dextran, glutamate or glucose), proteins (such as dried milk serum, albumin or casein) or degradation products thereof. Suitable buffers are for example alkali metal phosphates. Suitable preservatives are thimerosal, merthiolate and gentamicin. Diluents include water, aqueous buffer (such as buffered saline), alcohols and polyols (such as glycerol).

15 If desired, the live vaccines according to the invention may contain an adjuvant. Examples of suitable compounds and compositions with adjuvant activity are the same as mentioned below for the preparation of inactivated vaccines.

Although administration by injection, e.g. intramuscular, subcutaneous of the live vaccine according to the present invention is possible, the live vaccine is preferably administered by the  
20 inexpensive mass application techniques commonly used for avian reovirus vaccination. These techniques include drinking water and spray vaccination.

Alternative methods for the administration of the live vaccine include in ovo, eye drop and beak dipping administration.

25 In a preferred embodiment the present invention provides a vaccine against enteric disease conditions, such as those observed with MAS, comprising the avian reovirus in an inactivated form. The major advantage of an inactivated vaccine is the elevated levels of protective antibodies of long duration that can be obtained. This property makes such an inactivated vaccine in particular suited for breeder vaccination.

30 The aim of inactivation of the viruses harvested after the propagation step is to eliminate reproduction of the viruses. In general, this can be achieved by chemical or physical means. Chemical inactivation can be effected by treating the viruses with, for example, enzymes,

formaldehyde,  $\beta$ -propiolactone, ethylene-imine or a derivative thereof. If necessary, the inactivating compound is neutralised afterwards. Material inactivated with formaldehyde can, for example, be neutralised with thiosulphate. Physical inactivation can preferably be carried out by subjecting the viruses to energy-rich radiation, such as UV light or  $\gamma$ -rays. If desired, after treatment the pH can be adjusted to a value of about 7.

A vaccine containing the inactivated avian reovirus can, for example, comprise one or more of the above-mentioned pharmaceutically acceptable carriers or diluents suited for this purpose.

Preferably, an inactivated vaccine according to the invention comprises one or more compounds with adjuvant activity. Suitable compounds or compositions for this purpose include aluminium hydroxide, -phosphate or -oxide, oil-in-water or water-in-oil emulsion based on, for example a mineral oil, such as Bayol F® or Marcol 52® or a vegetable oil such as vitamin E acetate, and saponins.

Inactivated vaccines are usually administered parenterally, e.g. intramuscularly or subcutaneously.

The vaccine according to the invention comprises an effective dosage of the avian reovirus as the active component, i.e. an amount of immunising avian reovirus material that will induce immunity in the vaccinated birds or their progeny against challenge by a virulent virus. Immunity is defined herein as the induction of a significant higher level of protection in a population of birds after vaccination compared to an unvaccinated group.

Typically, the live vaccine according to the invention can be administered in a dose of  $10^2$ - $10^9$  TCID<sub>50</sub> per bird, preferably in a dose ranging from  $10^2$ - $10^6$  TCID<sub>50</sub>, and an inactivated vaccine may contain the antigenic equivalent of  $10^4$ - $10^{10}$  TCID<sub>50</sub> per bird.

Although, the avian reovirus vaccine according to the present invention may be used effectively in chickens, also other poultry such as turkeys, guinea fowl and quail may be successfully vaccinated with the vaccine. Chickens include broilers, reproduction stock and laying stock.

Because enteric disease conditions, such as those observed with MAS have been reported primarily in broiler chickens, the present invention preferably provides a vaccine for use in the protection of broilers against enteric disease conditions, such as those observed with MAS.

The age of the animals receiving a live or inactivated vaccine according to the invention is  
5 the same as that of the animals receiving the presently commercially available live- or inactivated avian reovirus vaccines. For example, broilers may be vaccinated directly from one-day-old onwards with the live attenuated vaccine according to the invention. Vaccination of parent stock, such as broiler breeders, can be done with a live attenuated or inactivated vaccine according to the invention or combinations of both. The advantages of this type of  
10 immunisation programme includes the immediate protection of one-day-old progeny provided by maternally derived antibodies vertically transmitted to the young birds. A typical breeder vaccination programme includes the vaccination of the breeders at 6-weeks of age with a live attenuated vaccine, followed by a vaccination between 14-18 weeks of age with an inactivated vaccine. Alternatively, the live vaccination may be followed by two vaccinations with  
15 inactivated vaccines on 10-12 weeks and 16-18 weeks of age.

The invention also includes combination vaccines comprising, in addition to the avian reovirus according to the invention, one or more vaccine components of other pathogens infectious to poultry. With such other pathogens infectious to poultry also avian reoviruses are  
20 meant which are antigenically distinct from the avian reoviruses according to the present invention, and include the avian reovirus strains associated with tenosynovitis.

Preferably, the vaccine components in the combination vaccine are the live attenuated or inactivated forms of the pathogens infectious to poultry.

In particular, the present invention provides a combination vaccine wherein all of the  
25 vaccine components are in an inactivated form.

Preferably, the combination vaccine comprises one or more (inactivated) vaccine strains of infectious bronchitis virus (IBV), Newcastle disease virus (NDV), infectious bursal disease virus (IBDV), fowl adenovirus (FAV), EDS virus and turkey rhinotracheitis virus (TRTV).

In the framework of the present invention the following micro-organism and hybridoma cell lines have been deposited at the European Collection of Animal Cell Cultures (ECACC), Salisbury, UK, on January 14, 1999:

5

virus/hybridoma	accession no.
avian reovirus ERS	99011475
INT 13-06	99011472
INT 14-11	99011473
15-01 INT	99011474

EXAMPLESExample 1.

5

**Characterisation of the novel avian reoviruses**AIsolation of the novel avian reoviruses.

Intestine and/or liver were individually isolated from chickens having digestive problems and wet litter resulting in growth retardation. The organs were individually homogenised in a homogeniser using glass pearls (2 mm) and PBS with antibiotics, for 20 min at maximum speed. Thereafter, the homogenised tissues were centrifuged. Intestine homogenate was centrifuged at 4000 rpm, and liver was centrifuged at 1200 rpm both for 15 minutes. Next, the supernatants were filtrated by pressing through filters with decreasing pore size (5, 1.2, 0.45, 0.2- $\mu$ m). One hundred  $\mu$ l of suspension passing through the 0.2- $\mu$ m filter was added to freshly prepared primary chicken embryo liver (CEL) cells present in tissue culture flasks. Four to 8 days after incubation the monolayers were inspected for the presence of a cytopathic effect (CPE). If no CPE was present the monolayers were frozen at -20°C, after 24h the monolayers were thawed. Next, 1 ml of this freeze/thawed suspension was added to freshly prepared CEL cells. If no CPE was visible after 4-8 days the culture was considered negative for virus growing on CEL cells. If after the first passage or the second passage CPE was observed, then the virus was further characterised by the plaque reduction assay and immuno-fluorescence-technique (IFT).



**B****In vitro characterisation of the novel avian reoviruses with the plaque reduction assay****1. Production of antiserum against different avian reovirus strains**

5

**Strain ERS (live):**

Ten, 3 weeks old SPF chickens were subcutaneously infected with  $10^{5.8}$  TCID<sub>50</sub>/animal of strain ERS (isolate-2). Three weeks after infection blood was collected and serum isolated and animals were again infected  $10^{5.6}$  TCID<sub>50</sub>/animal. Two weeks after the second infection  
10 blood was collected and serum isolated. After acquisition, all sera were heat inactivated 56°C, 30 min and stored in small aliquots at -20°C.

**Strain 2177 (live):**

Fifteen, 4 weeks old SPF chickens were subcutaneously infected with  $10^{4.2}$   
15 TCID<sub>50</sub>/animal. Four weeks after infection blood was collected and serum isolated. After acquisition, all sera were heat inactivated 56°C, 30 min and stored in small aliquots at -20°C.

**Strains 1733 and 2408 (inac):**

Twelve, 4 weeks old SPF chickens were intramuscularly inoculated with one of the avian  
20 reovirus strains 1733 or 2408 adjuvated with a w/o-emulsion. Animal dose: 490 Elisa Units/animal; representing an infectivity titre before inactivation between  $10^{7.0}$ - $10^{10.0}$  TCID<sub>50</sub>/animal. Four weeks after infection blood was collected and serum isolated. After acquisition, all sera were heat inactivated 56°C, 30 min and stored in small aliquots at -20°C.

**25 Inactivated commercially available vaccines containing avian reovirus strains:**

Ten, 3-4 weeks old SPF chickens were intramuscularly or subcutaneously inoculated with one of the following commercially available inactivated avian reovirus vaccines: ISBI, Fort Dodge; Kaketsuken and Intervet International BV. Three weeks after infection blood was collected and serum isolated. After acquisition, all sera were heat inactivated 56°C, 30 min and  
30 stored in small aliquots at -20°C.

The sera prepared above were used in the IFT and plaque reduction assay.

## 2. Immunofluorescence test (IFT)

The IFT is carried out essentially as described in paragraph C. below. Briefly, Vero cells were grown in 96-well polystyrene microtitre plates until confluence. Different monolayers were inoculated with Reovirus strain 1133. 100 µl of chicken serum was added to the first well of the plate. Serial 3-fold dilutions are made. After the incubation and washing steps, the plates were reacted with 1:100 diluted fluorescent isothiocyanate-labelled goat-anti-chicken. The presence of fluorescence was observed with a fluorescence microscope. The titre (quality of the serum) is determined by end point dilution. This is the highest dilution of serum that is still able to induce a clear fluorescence signal. In Table 1 the results of the IFT for sera used in the plaque reduction test are shown:

Table 1

Serum against avian reovirus strain	End dilution (expressed in log2)
2177	2025 (11.0)
ERS	6075 (12.6)
1733	6075 (12.6)
2408	18225 (14.2)
Arvax	75 (6.2)
Nobilis Reo	18225 (14.2)
Tri Reo	6075 (11.6)
Oilvax Reo	18225 (14.2)
Negative serum	<3 (<1)

Nobilis Reo™ commercially available from Intervet International b.v.

Arvax™ commercially available from ISBI

Tri Reo™ commercially available from Fort Dodge

Oilvax Reo™ commercially available from Kaketsuken

### 3. Plaque reduction assay

Antiserum of appropriate quality should be used in the plaque reduction assay to determine the antigenic relationship between avian reoviruses belonging to the new antigenic class and to distinguish the new avian reoviruses from known avian reoviruses (all but the  
5 Arvax antiserum fulfilled the required quality of the antiserum). ERS antiserum was raised against live ERS isolate 1, and live and inactivated ERS isolate 2.

Freshly prepared CELs were resuspended in tissue culture medium supplemented with 5% fetal calf serum and antibiotics to a final concentration of  $1 \times 10^6$  cells per millilitre. Sixty-mm tissue culture dishes were filled with 5-ml cell suspension and incubated at 37°C for 24h.

10 The next day the virus to be investigated were diluted in plastic tubes in medium with antibiotics. Dilutions from  $10^{-1}$  till  $10^{-7}$  were prepared. Next 200 µl of each dilution was mixed with 50 µl of the serum to be tested. This mixture was incubated at 37°C for 1h. As negative control 200 µl of virus dilution was mixed with 50 µl of medium.

The medium on top of the monolayers present in the 60-mm tissue culture dishes was  
15 discarded. Next, 100 µl of the different virus mixtures (with or without serum) is added onto a confluent monolayer. For each virus mixture at least 2 monolayers (dishes) were infected. The infected monolayers were incubated for 1h at 37°C. Thereafter, the infected monolayers were covered with 5 ml agar-solution (containing medium, FCS and antibiotics; final agar concentration 3.0%; final FCS concentration 2.5%). The dishes were incubated for 4 days at  
20 37°C. Next to each tissue culture dish 2-ml neutral red solution (0.02%) was added. After 4h of incubation at 37°C the number of plaques were counted per dish. Only the tissue culture dishes that contained 150 or less plaques were counted.

Plaque reduction is calculated as follows: the number of plaques of a certain virus at a certain dilution without serum is set at 100%. This is then compared to the number of plaques at  
25 the same virus dilution but with serum.

The results of a first experiment with different viruses and sera tested are given in Table 2A. Table 2B shows the result of a second experiment with the viruses and sera as indicated. The serum against the inactivated ERS vaccine was raised as described for the inactivated 1733/2408 strains, except that blood was collected 5 weeks after vaccination.

Table 2A

Virus	Serum								
	1733	2408	2177	ERS-2	Nobilis Reo	Arvax	Tri Reo	Oilvax Reo	neg
	inac	inac	live	live	inac	inac	inac	inac	-
ERS-1	0*	0	0	89					-
ERS-2	0	0	0	91	0	0	0	0	-
ERS-3	0			100					-
1733	91			85					-
2408		91		87					-
2177			85	83					-
K255	91		88	98					-

\* % plaque reduction

- reference

5

Table 2B

Virus	Serum			
	ERS-2 live	ERS-1 live	inactivated ERS vaccine	1133 live
ERS-2	95*	81	89	12
ERS-1	100	100	nd	nd

\* % plaque reduction

nd = not done

10

CIn vitro characterisation of the novel avian reoviruses with IFT: antiserum panel reaction pattern

Polyclonal antiserum was prepared by infecting rabbits (1-1,5 kg) with purified avian  
 5 reovirus strain 1133. Booster injections took place 28 and 84 days after the first infection. Blood was collected and serum isolated 14 days after the last injection.

The different avian reovirus strains were characterised with different Moabs. Primary CEL cells were grown in 96-well polystyrene microtitre plates. Uninfected cells served as controls. After 2-4 days of incubation at 37°C with 5% CO<sub>2</sub>, infected monolayers were fixed with cold 96%  
 10 ethanol. The alcohol was discarded and the plates were washed with washing buffer and 100 µl of different hybridoma cell culture supernatant diluted 1:50 or 1:200 in PBS or 100 µl of rabbit polyclonal serum (rabbit 68A) diluted 1:50, was added to each well. The plates were incubated for 60-90 min at 37°C, washed twice with washing buffer and reacted with 1:100 diluted fluorescent isothiocyanate-labelled rabbit-antimouse- or 1:100 diluted isothiocyanate-labelled  
 15 goat-anti-rabbit serum. Finally, the plates were washed and fixed with a glycerol/PBS solution (1:1). The presence of fluorescence was observed with a fluorescence microscope.

The antiserum panel used in this experiment consisted of the following polyclonal antiserum and Moabs raised against the prototype avian reovirus strain 1133:

20	Rabbit 68A	rabbit polyclonal antiserum
	Moab 154	Vakharia et al. 1996 (supra)
	Moab 14-67	Intervet International B.V.
	Moab INT 13-06	ECACC accession no. 99011472
	Moab INT 14-11	ECACC accession no. 99011473
25	Moab 15-01 INT	ECACC accession no. 99011474

Avian reovirus isolates obtained by the method described above were further characterised by their reactivity with this antiserum panel. The new reovirus isolates have a distinct reaction pattern with the panel of poly- and monoclonal antibodies. The hitherto known avian reovirus  
 30 strains (S-1133 to CO8) isolated from field cases of MAS and tenosynovitis do not react according to the new pattern (see Table 3).

New reaction pattern Positive: polyclonal rabbit 68A, 154, 14-67

Negative: INT 13-06, INT 14-11, 15-01 INT

5 Table 3

	Rabbit 68A	154	14-67	INT 14-11	INT 13-06	15-01 INT					
Virus											
S-1133											
1133											
2408											
UM 203											
WVU 1675 Olson											
6261											
Enterovax™											
Tensynovac™											
1733											-
2177										-	-
206691				-	-						
CO8				-							
ERS isolates											
ERS-1				-	-	-					
ERS-2				-	-	-					
ERS-3				-	-	-					
ERS-4				-	-	-					
ERS-5				-	-	-					
ERS-6				-	-	-					
ERS-7				-	-	-					
ERS-8				-	-	-					
ERS-9				-	-	-					
ERS-10				-	-	-					
ERS-11				-	-	-					
ERS-12				-	-	-					
ERS-13				-	-	-					

Enterovax™ and Tensynovac™ are avian reovirus vaccines commercially available from Schering-Plough Animal Health and Intervet Inc.

DIn vivo characterisation of the novel avian reoviruses5 Experimental infection with plaque purified avian reovirus ERS

## Experiment 1

30 One-day old SPF-chickens were orally infected with plaque purified reovirus ERS (isolate 1). Four, 7 and 10 days after infection livers from 10 animals were isolated and investigated for  
10 microscopic lesions.

## Experiment 2

30 One-day old broilers with maternal antibodies against reovirus were orally infected with plaque purified reovirus strain ERS. Seven and 10 days after infection animals were observed for clinical signs. Special attention was paid to wet litter

## 15 Experiment 3

30 One-day old broilers with maternal antibodies against reovirus were orally infected with plaque purified reovirus strain ERS. One, 2 and 4 weeks after infection 10 animals per group were weighed to investigate growth retardation.

## Experiment 4

20 Fifteen, one-day old broilers with maternal antibodies against reovirus per group were orally or subcutaneously infected with plaque purified reovirus strain ERS (isolate-2), at day old or at 1 week old. Fifteen animals of the same age and source were not infected and served as negative control. Animals were weighed each week for a period of 7 weeks to investigate growth retardation.

25

Results

## Experiment 1

One-day old infected SPF-chickens that were orally infected with the reovirus showed multifocal vacuolation of hepatocytes and/or Kupffer cells, 4 to 10 days after infection.

**Experiment 2**

Ten days after oral infection, broilers showed enteritis resulting in too liquid faeces observable as clothing of pasting around the cloaca of the birds in contrast to a non-infected broiler control group of the same age and source held under identical conditions.

5

**Experiment 3**

One-day-old orally infected broilers had a weight of 121.9, 327.0 or 913.1 g. at the age of 1, 2 or 4 weeks old. In contrast, non-infected broilers of the same age and source housed under identical conditions weighed 134.8, 337.6 or 999.9 g., at the age of 1, 2 or 4 weeks old.

10 **Experiment 4**

Results are depicted in figure 2A and 2B. At the age of 7 weeks animals infected at day old via the oral or subcutaneous route showed a growth retardation of approximately 34% compared to the non-infected control animals. At 7 weeks of age, the ratio in weight between non-infected controls animals versus infected animals was 2469g versus 1635g.

15 At the age of 7 weeks animals infected at one week old via the oral or subcutaneous route showed a growth retardation of approximately 25% compared to the non-infected control animals. At 7 weeks of age, the ratio in weight between non-infected control animals versus infected animals was 2469g versus 1842g.

In conclusion, the reovirus strain ERS is capable of inducing growth retardation.

20

**Example 2****Animal vaccination studies****A**25 **Preparation of an inactivated avian reovirus vaccine**

Primary CEL cells were prepared at a final concentration of  $1 \times 10^6$  cells/ml. The cells were cultured in Eagles MEM containing 0.1% antibiotics and 5% foetal calf serum. To 25 ml of this cell suspension 0.1 ml of reovirus isolate ERS (isolate 1) was added. After incubation for 5 days in a high-humidity incubator at 37°C, CPE was clearly visible and the monolayer completely destroyed. The infectious titre of the infected cell suspension was 4.2 log<sub>10</sub> TCID<sub>50</sub>/ml. The reovirus was inactivated by adding formaldehyde to the infected cell suspension to an end concentration of 0.2%. The suspension was incubated at 37°C for 48h.

30



Thereafter, the formaldehyde was neutralised by an equimolar amount of sodiumbisulfite. The inactivated reovirus was used to prepare different inactivated reovirus vaccines. The inactivated reovirus suspension was mixed with a mineral oil phase in a ratio of 45:55 (w/o emulsion).

## 5 Vaccination

SPF animals of 3-4 weeks old were intramuscularly vaccinated with the inactivated reovirus vaccines (0.5ml/animal). Two, 4 and 6 weeks after vaccination serum was investigated for the presence of antibodies against reovirus. Six weeks after vaccination animals were challenged via the footpad route. Challenge virus used was the pathogenic homologous reovirus strain (2.2 log TCID<sub>50</sub>/animal). The degree of inflammation and discoloration of the footpad and shank was scored for a period of 2 weeks. A chicken was considered protective against challenge if the footpad inflammation cumulative value was less than the mean of the footpad inflammation of the non-vaccinated challenged controls minus two standard deviations.

In a similar study as outlined above, the animals were challenged at five weeks of age and the protection against severe challenge was evaluated by means of mortality, morbidity and footpad lesions

## Results

The protection data are depicted in Figure 1A and 1B. The vaccinated chickens showed a footpad inflammation cumulative value which is significantly reduced compared to the control group, indicating that the vaccinated chickens were protected against severe reovirus challenge. The protection data based on mortality and morbidity also demonstrate a significant protection against severe challenge.

## B

### Vaccination

SPF animals of 3-4 weeks old were intramuscularly vaccinated with inactivated ERS reovirus vaccine (isolate 2) similar to that described above and revaccinated 6 weeks after the first vaccination. Not vaccinated animals of the same age and source served as control. Two, 4, 5, 6 weeks after the first vaccination and 2 weeks after the second vaccination sera were investigated for the presence of antibodies against Reovirus with the Idexx ELISA. Six weeks after the first vaccination or 2 weeks after the second vaccination animals were challenged via the footpad route with pathogenic Reovirus strain ERS. The average lesion score of the degree of inflammation and discoloration of the footpad and shank was measured each day for a period of 14 days after challenge.

### Results

The serological response induced by the inactivated reovirus ERS vaccine increased in the first 4 weeks after vaccination and levelled off between 5 and 6 weeks after the first vaccination. The second vaccination resulted in an increase from approximately 10 log<sub>2</sub> to 12 log<sub>2</sub>, 2 weeks after the second vaccination (figure 3A). In figure 3B the data are shown of vaccinated and non-vaccinated chickens that were challenged 5 weeks after the first vaccination or 2 weeks after the second vaccination. All vaccinated chickens showed a significant decrease of lesions in the footpad and shank compared to non-vaccinated birds. All vaccinated chickens showed footpad inflammation cumulative values less than the mean of the footpad inflammation of the non-vaccinated, challenged controls minus two standard deviations, indicating that all animals were protected against severe REO challenge. Some mild footpad swellings were observed in the vaccinated group, however these lesions were of a temporary nature. Furthermore, the temporary footpad lesions were less in the animals that were vaccinated twice when compared to animals that were vaccinated once.

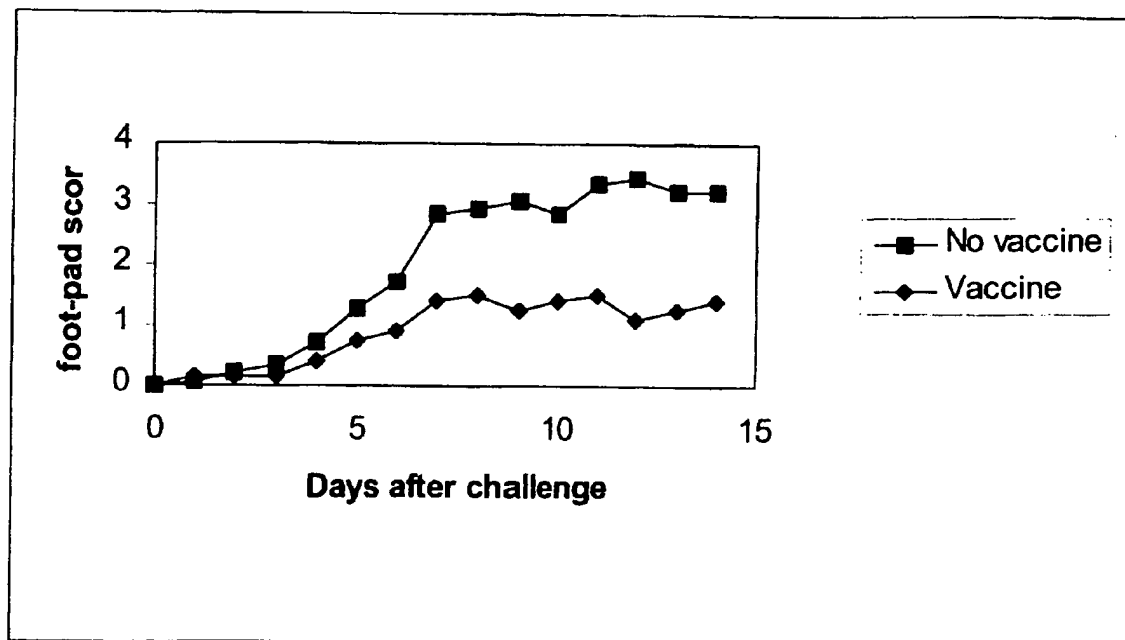
CLAIMS

- 1 An avian reovirus belonging to an antigenic class of avian reoviruses, characterised in that the avian reovirus belonging to the antigenic class is able to induce antiserum in an animal, which antiserum causes a reduction of the plaques formed by avian reovirus ERS, a sample of which is deposited at the ECACC under accession no. 99011475, of at least 75% in a plaque reduction assay.
- 2 An avian reovirus according to claim 1, characterised in that the induced antiserum against the avian reovirus is able to cause a reduction of the plaques formed by avian reovirus ERS, a sample of which is deposited at the ECACC under accession no. 99011475, of at least 80 %, preferably at least 90%, in a plaque reduction assay.
- 3 An avian reovirus according to claim 1 or 2, characterised in that the avian reovirus positively reacts with polyclonal avian reovirus antiserum but not with monoclonal antibodies samples of which are deposited at the ECACC under accession no. 99011472, 99011473 and 99011474.
- 4 An avian reovirus according to claim 3, characterised in that it is avian reovirus ERS, a sample of which is deposited at the ECACC under accession no. 99011475.
- 5 A vaccine for use in the protection of poultry against disease conditions resulting from an avian reovirus infection, comprising an avian reovirus according to claims 1-4, and a pharmaceutical acceptable carrier or diluent.
- 6 A vaccine according to claim 5, characterised in that the avian reovirus is in a live attenuated form.
- 7 A vaccine according to claim 5, characterised in that the avian reovirus is in an inactivated form.

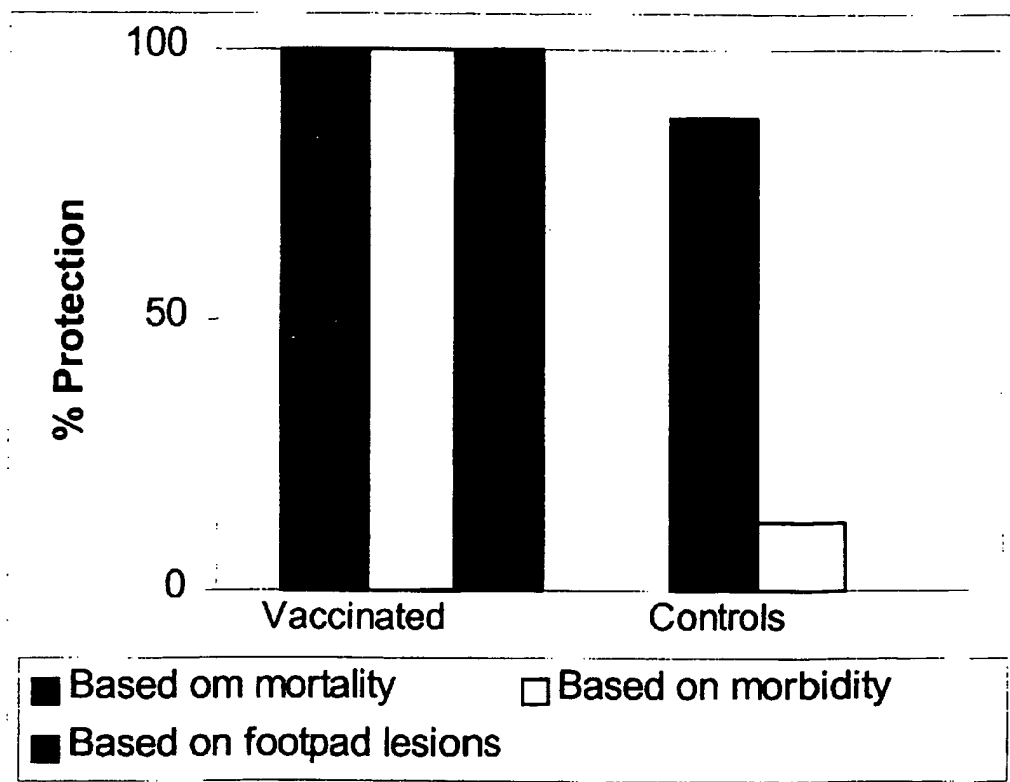
- 8 A vaccine according to claims 5-7, characterised in that the vaccine further comprises an adjuvant.
- 9 A vaccine according to claims 5-8, characterised in that the vaccine further comprises one or more vaccine components of other pathogens infectious to poultry.
- 10 A method for the preparation of avian reoviruses defined in claims 1-4 comprising the steps of:
- a. inoculating a susceptible substrate with the avian reovirus,
  - b. propagating the avian reovirus, and
  - c. harvesting avian reovirus containing material.
- 11 A method for the preparation of a vaccine for use in the protection of poultry against disease conditions resulting from an avian reovirus infection, comprising combining the harvested reoviruses obtained by the method according to claim 10, if desired after inactivation of the reoviruses, with a pharmaceutical acceptable carrier or diluent.
- 12 A method for controlling disease conditions resulting from an avian reovirus infection in poultry, comprising administering the vaccine according the claims 1-4 to the birds.

Figure 1

A: Protection evaluated by footpad lesions



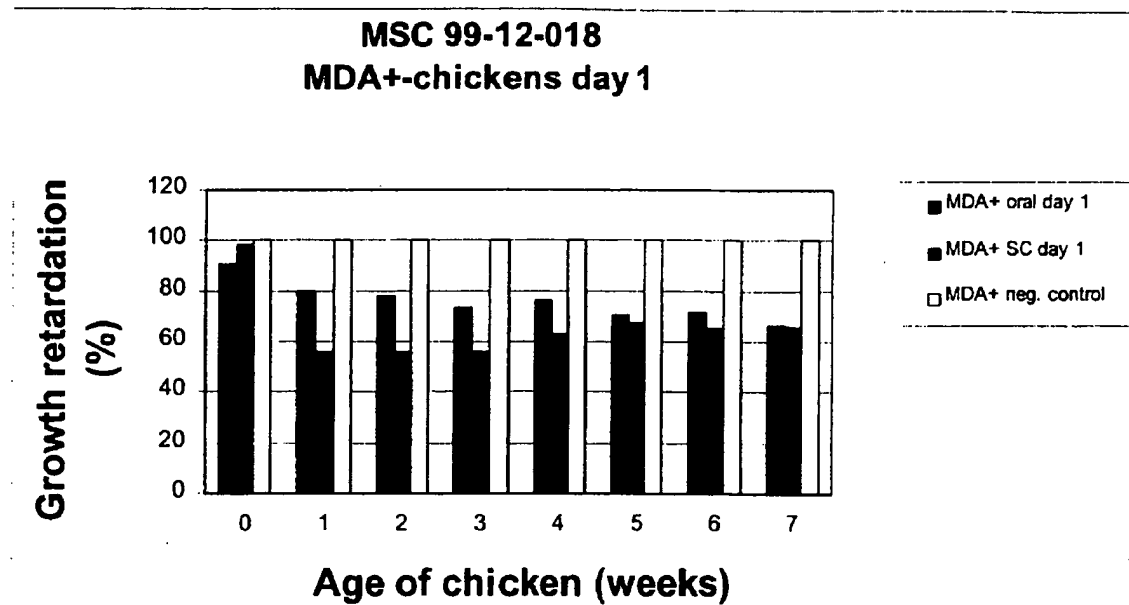
5 B: Protection evaluated by mortality, morbidity and footpad lesions.



28

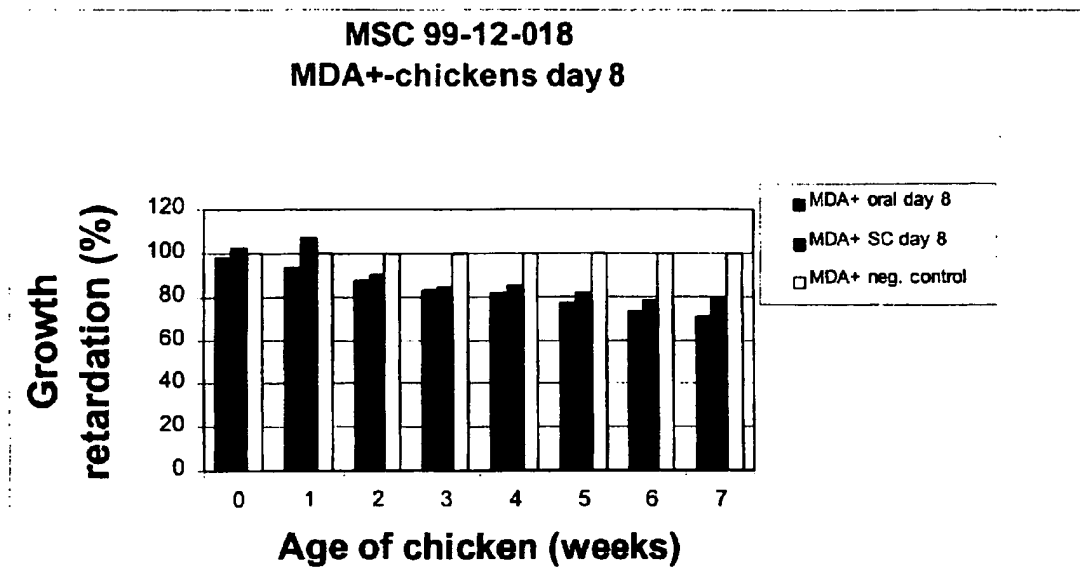
Figure 2

A: Growth retardation of broilers infected with ERS at day-old.



5

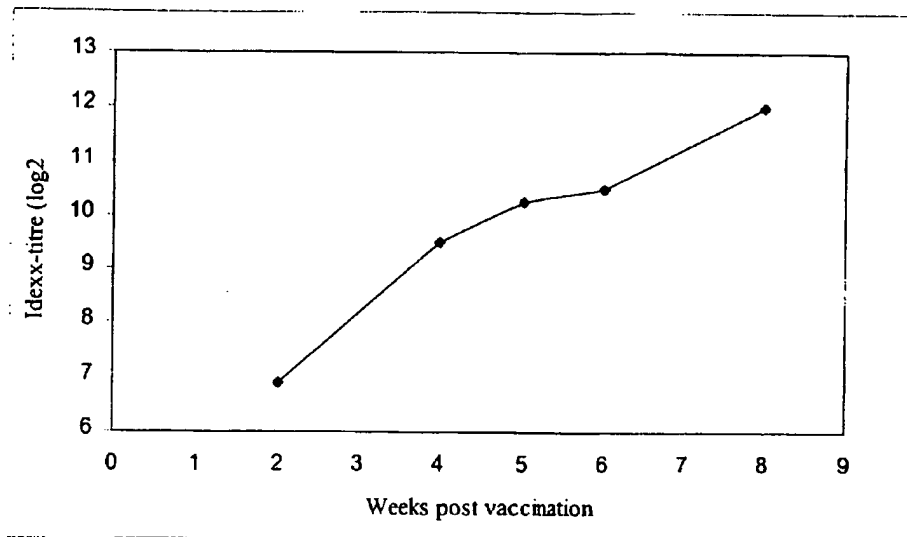
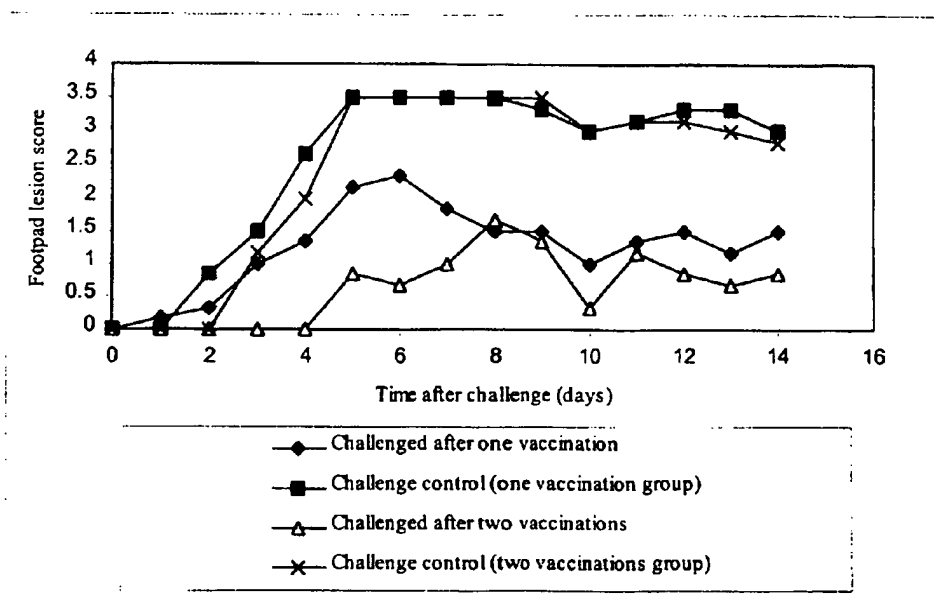
B: Growth retardation of broilers infected with ERS at 8 days-old.



10

**Figure 3****A: Serological response after vaccination.**

5

**B: Footpad lesion score.**

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☒ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**